Ultrastructural Changes, Nuclear Factor-κB Activation, and Tumor Necrosis Factor-α Expression in Brain after Acute Normovolemic Hemodilution and Controlled Hypotension in Rats

Ran Lv¹, Wei Zhou², Manlin Duan³, Yali Ge⁴, Taidi Zhong¹

¹Department of Anesthesiology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China
²Department of General Surgery, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China
³Department of Anesthesiology, Jinling Hospital, Nanjing, China
⁴Department of Anesthesiology, Subei People’s Hospital, Medical College, Yangzhou University, Yangzhou, China

Aim To examine brain damage following different degrees of acute normovolemic hemodilution combined with controlled hypotension (ANH-CH) by neuronal morphological analysis and investigate the expression of nuclear factor-kappa B (NF-κB) activity and tumor necrosis factor-alpha (TNF-α) in the rat.

Methods Forty rats were randomly assigned to receive a sham operation or ANH-CH (with hematocrit 30%, 25%, 20%, and 15%). ANH was performed after baseline physiological parameters had been monitored for 20 minutes. CH was induced 30 minutes later using sodium nitroprusside and mean arterial pressure was maintained at 50-60 mm Hg for 1 hour. Rats were euthanatized 3 and a half hours after operation. TNF-α levels and NF-κB activities in cerebral temporal cortex were measured. Ultrastructural alterations in the CA1 region of the rat hippocampi were observed. Changes in mitochondria were evaluated semi-quantitatively.

Results Marked ultrastructural alterations, such as mitochondrial denaturalization and nucleus distortion, were observed in the CA1 region of the hippocampus in the ANH-CH hematocrit 20% group and ANH-CH hematocrit 15% group. TNF-α expression and NF-κB activity in the cerebral temporal cortex significantly increased in all ANH-CH groups and peaked in the ANH-CH hematocrit 25% group.

Conclusion Severe ANH-CH with hematocrit ≤20% may induce cerebral damage and should be avoided. NF-κB activation and TNF-α expression may play a functional role under the ischemic condition. A better understanding of the role of NF-κB and TNF-α in the brain may lead to a novel approach for preventing and treating various neurological disorders.
Evidence suggests that restrained blood transfusion is associated with a low risk of morbidity and mortality, even in the elderly and critically ill patients (1). Both acute normovolemic hemodilution (ANH) and controlled hypotension (CH) have independently demonstrated their efficacy as blood-saving techniques (2,3). Consequently, their combination could produce an even better method for blood conservation. However, the combined use of ANH and CH is still underutilized, because of concerns that reduced oxygen content and perfusion pressure may lead to ischemic and hypoperfusion injury to vital organs (4). The vital organ most susceptible to hypoxia is the brain. It has high oxygen demand and exclusive dependence on glucose oxidation, which necessitates a tight control system for cerebral blood flow. ANH can increase cerebral blood flow to compensate for decreased arterial oxygen content (5), but ANH in combination with CH reduces the increased cerebral blood flow (4). Therefore, care must be taken to avoid brain damage when this combined technique is used.

Nuclear factor-kappa B (NF-κB) is a transcription factor expressed in all cell types in the nervous system. It can be activated by many different physiological stimuli. Previous studies have shown that NF-κB could activate gene expression in response to ischemia, which plays a pivotal role in neuronal survival (6). However, it has been reported that NF-κB activation may be associated with apoptotic or necrotic cell death (7).

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine that may play an important role both in normal central nervous system development and in the response of the brain to diverse forms of injury (8). Recent data suggest that it may be synthesized and secreted by several central nervous system cell types including microglia, astrocytes, and neurons. Some authors consider that TNF-α may be of extreme benefit in repair and regeneration of brain ischemic injury (9). On the other hand, data also show that it may be an important mediator in neuronal apoptosis (10). However, no previous studies have evaluated whether ANH-CH stimulates TNF-α expression or NF-κB activation in the brain of rats. To address this question, we aimed to determine whether ANH-CH causes cerebral hypoxia and to evaluate the role of NF-κB and TNF-α in the brain ischemic injury following ANH-CH.

**Material and methods**

**Animals and experimental protocol**

Adult male Sprague-Dawley rats (350-400 g; Shanghai Animal Center, Shanghai, China) were kept in accordance with the Institutional Animal Care Committee guidelines in a room with a temperature of 22°C and a 12-hour light/dark cycle. Animals were allowed at least 7 days to acclimatize before experiments. They were anaesthetized by intraperitoneal administration of 1250 mg/kg urethane (Shanghai Chemical Reagent Co, Shanghai, China), intubated, and mechanically ventilated with a volume-control mode (tidal volume 8 mL/kg, frequency 60 bpm, FiO2 1.0). From blood-gas analysis (Radiometer ALB 500, London Scientific, London, UK), ventilation parameters were adjusted to maintain arterial oxygen saturation (SaO2) greater than 95% and PaCO2 within a normal physiological range.

The tail vein was cannulated with a polyethylene catheter for intravenous administration of solutions. The macrohemodynamic parameters including mean arterial blood pressure (MAP), heart rate (HR), and central venous pressure (CVP) were measured on the left femoral artery and vein, which were catheterized with a microtip transducer (Abbott Laboratories Ltd, Chicago, IL, USA).
Forty male rats were randomly divided into 5 treatment groups (8 rats per group) – sham-operated group and four groups with different degrees of ANH-CH (hematocrit 30%, 25%, 20%, and 15%). After 20 minutes of monitoring of baseline parameters, ANH was accomplished by withdrawal of blood from the femoral artery and simultaneous administration of equivalent volume of hydroxyethyl starch (130/0.4, 6%; medium molecular weight, low degree of substitution; Fresenius Kabi, Bad Homburg, Germany) through the tail vein at the same rate. To reach different hematocrit targets, the volume (V) of blood to be removed was calculated as follows (11): V = EBV × (Hi-Hf)/Hav, where EBV is the estimated blood volume (70 mL/kg), Hi the initial hematocrit, Hf the final hematocrit after ANH, and Hav mean hematocrit (mean of Hi and Hf). Thirty minutes after ANH, CH was induced with infusion of 0.01% sodium nitroprusside at 0.15-15 μg kg⁻¹ min⁻¹ to decrease MAP to 50-60 mm Hg in 5 minutes and maintain for 1 hour. The sham-operated group was anesthetized and underwent the same anesthetic and surgical technique, but without ANH and CH. MAP, HR, CVP, and arterial blood gas were recorded immediately before hemodilution (T0), just before CH (T1), in the middle of controlled hypotension (T2), at the end of CH (T3), and 30 minutes after recovery from CH (T4) (Figure 1). After operation lasting for 3.5 hours, animals were sacrificed by an overdose of urethane. During the experiment, a heating pad and heating lamp were used to maintain the rectal temperature of rats at about 37°C. The total doses of sodium nitroprusside did not differ among the treated groups, and the need for additional doses of urethane used to kill the rats was similar. The study protocols were approved by the Institutional Animal Care Committee of Sir Run Run Shaw Hospital.

**Histological and ultrastructural studies**

With the animals still anesthetized, systemic circulation was perfused with 0.9% saline. The cerebral cavity of rats was carefully opened, the hippocampus was immediately removed, and a 500-μm block (measured along the pyramidal cell body layer) of area CA1 was dissected and fixed for 48 hours in Somogyi’s solution of paraformaldehyde, glutaraldehyde 25%, and picric acid (pH 7.4) for electron microscopy (12). The tissue was dehydrated in ethanol and embedded in epoxy resin for ultrastructural studies.

Changes in mitochondria were evaluated semiquantitatively (13,14), whereas changes in other cell organelles and components were simply described. Electron micrographs were taken of each of the five biopsies, and in each micrograph 20 mitochondria were selected at random. Each mitochondrion was graded on a scale of 0 to 3, as follows: 0 – normal structure (Figure 2A); 1 – a little change but clear crests (Figure 2B); 2 – edematous change (Figure 2C); 3 – accumulation of amorphous material (Figure 2D).

**Determination of cerebral cortex cytokine levels**

Cerebral temporal cortex tissues were homogenized in extract buffer (0.32 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L Tris [pH 7.4], 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μmol/L leupeptin, and 1 mmol/L β-mercaptoethanol), and 0.1% (wt/vol) proteinase inhibitor. Each homogenate was centrifuged at 1000 g for 10 minutes and the pellet was discarded. The supernatant was centrifuged at 160 000 rpm for 30 minutes and the supernatant collected (15). All procedures were performed at
Protein concentrations were determined by Bradford assay. The samples were kept frozen at -70°C until assay. The levels of TNF-α in cerebral temporal cortex tissue were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for the rat cytokines, according to the manufacturer’s instructions (Diaclone Research, Besancon, Cedes, France). Each measurement was performed in duplicate according to the manufacturer’s recommendations and the averages were reported. Values were expressed as pg/mg protein.

**Determination of cerebral cortex NF-κB activities by EMSA**

The nuclear extracts were prepared from cerebral temporal cortex tissue according to a method established by our laboratory (16) and kept at -70°C for electrophoretic mobility shift assay (EMSA). Protein concentrations were determined using Bradford protein assay formats. EMSA was performed using a commercial kit (Promega, Madison, WI, USA). NF-κB consensus oligonucleotide (5′-AGTT-GAGGGACTTCCAGGC-3′) was labeled with [γ-32P] ATP (Free Biotech, Beijing, China) with T4 polynucleotide kinase. Equal amounts of nuclear extract (80 µg) were added to 9 µL of gel shift binding buffer (Tris-HCl 10 mmol/L [pH 7.5], NaCl 50 mmol/L, EDTA 0.5 mmol/L, MgCl2 1 mmol/L, DTT 0.5 mmol/L, 4% glycerol, 0.05 g/L Poly dIdC; 15 minutes, room temperature). The mixture was incubated for 30 minutes with 1 µL of the 32P-labeled oligonucleotide probe. One microliter of loading buffer was added to stop the reaction and the sample was electrophoresed in a 4% polyacrylamide gel. The dried gel was exposed to x-ray film (Fuji Hyperfilm) at -70°C. The intensity of the NF-κB complex was quantified by densitometry.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD). Analyses were performed by Statistical Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA). Differences among groups were determined by one-way analysis of variance (ANOVA), followed by the least-significant-difference (LSD) post hoc test. Significant differences within groups by time as compared with the baseline were tested by the repeated measures analysis. Differences were considered statistically significant if P<0.05.

**Results**

**Effect of ANH-CH on macrohemodynamics and arterial blood gas parameters**

MAP, HR, and CVP were similar in all groups at baseline. MAP and HR did not differ between T0 and T1 in any group. With ANH-CH, HR was increased at T2 and T3, and MAP was lower at T4 than at baseline. Throughout the experiment, CVP was stable. PaO2 and PaCO2 were within the reference range at all time points, but pH decreased sig-
Acidity:

PaCO2 (kPa):  

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Effect of ANH-CH on ultrastructural changes in the hippocampus

ANH-CH hematocrit 20% group and ANH-CH hematocrit 15% group showed uniform alterations in the cerebral ultrastructure. Mean mitochondrial scores in the two groups were significantly higher than in the sham-operated group (P<0.003; P<0.001) (Figure 3). Most nuclei appeared normal in the sham-operated group (Figure 4A). ANH-CH hematocrit 30% significantly at T3 (P=0.007 vs baseline) and T4 (P=0.011 vs baseline; P=0.038 vs sham-operated group) in the ANH-CH hematocrit 15% group (Table 1).

Figure 3. Ultrastructural mitochondrial scores of hippocampal neurons in CA1 region following acute normovolemic hemodilution combined with controlled hypotension (ANH-CH). S – sham-operated group; 30% – ANH-CH hematocrit 30% group; 25% – ANH-CH hematocrit 25% group; 20% – ANH-CH hematocrit 20% group; 15% – ANH-CH hematocrit 15% group. Values are presented as means±SD. Asterisk indicates P<0.01 vs sham-operated group at the end of the experiment.
According to the image, the document contains the following content:

**Effect of ANH-CH on the level of TNF-α and activity of NF-κB in the cerebral cortex**

Compared with the sham-operated group (6.4 ± 0.3 pg/mg protein), ANH-CH in other groups of rats was associated with a significant increase in TNF-α levels in the cerebral cortex tissue (ANH-CH hematocrit 30% group 8.5 ± 0.5 pg/mg protein [P<0.001], ANH-CH hematocrit 25% group 11.6 ± 0.6 pg/mg protein [P<0.001], ANH-CH hematocrit 20% group 8.2 ± 0.4 pg/mg protein [P=0.001], ANH-CH hematocrit 15% group 7.8 ± 0.5 pg/mg protein [P=0.005]), which achieved the maximum in the ANH-CH hematocrit 25% group (P<0.001 vs the other three groups) (Figure 5). Changes in cerebral activities of NF-κB were parallel to those in TNF-α levels. The NF-κB activity was expressed as the ratio between the photodensity of NF-κB and the photodensity of the background. In the four treatment groups, cerebral cortical NF-κB

(Figure 4B), and ANH-CH hematocrit 25% group (Figure 4C). Margination and clumping of chromatin were seen occasionally in the ANH-CH hematocrit 20% group, but more frequently in the ANH-CH hematocrit 15% group. Nuclear membrane was slightly irregular in the ANH-CH hematocrit 20% group (Figure 4D) and became more irregular in the ANH-CH hematocrit 15% group (Figure 4E). Glial activation, such as swollen glia assembling around the neurons, could be seen in the ANH-CH hematocrit 20% (Figure 4F) and ANH-CH hematocrit 15% group (Figure 4G).

![Image of nuclear changes in CA1 region of rat hippocampus after acute normovolemic hemodilution combined with controlled hypotension (ANH-CH).](image)

**Figure 4.** Nuclear changes in CA1 region of rat hippocampus after acute normovolemic hemodilution combined with controlled hypotension (ANH-CH). (A) Ultrastructure of representative nucleus in CA1 region of hippocampus in sham-operated group; (B) ultrastructure of representative nuclei in CA1 region of hippocampus in ANH-CH hematocrit 30% group; (C) ultrastructure of representative nuclei in CA1 region of hippocampus in ANH-CH hematocrit 25% group; (D) ultrastructure of representative nuclei in CA1 region of hippocampus in ANH-CH hematocrit 20% group; (E) ultrastructure of representative nuclei in CA1 region of hippocampus in ANH-CH hematocrit 15% group; (F) glial alteration (black arrow) in ANH-CH hematocrit 20% group; and (G) glial alteration (black arrow) in ANH-CH hematocrit 15% group.

![Image of Effect of acute normovolemic hemodilution combined with controlled hypotension (ANH-CH) on tumor necrosis factor-α (TNF-α) level in the cerebral cortex tissue.](image)

**Figure 5.** Effect of acute normovolemic hemodilution combined with controlled hypotension (ANH-CH) on tumor necrosis factor-α (TNF-α) level in the cerebral cortex tissue. S – sham-operated group; 30% – ANH-CH hematocrit 30% group; 25% – ANH-CH hematocrit 25% group; 20% – ANH-CH hematocrit 20% group; 15% – ANH-CH hematocrit 15% group. Values are presented as mean±SD. Asterisk indicates P<0.01 vs sham-operated group, and string indicates P<0.001 vs ANH-CH hematocrit 30% group, ANH-CH hematocrit 20% group, and ANH-CH hematocrit 15% group.

activity increased to 6.04 ± 0.52 (ANH-CH hematocrit 30% group; P<0.001), 8.62 ± 0.32 (ANH-CH hematocrit 25% group; P<0.001), 5.26 ± 0.38 (ANH-CH hematocrit 20% group; P=0.001), and 4.86 ± 0.41 (ANH-CH hematocrit 15% group; P=0.008) relative units, respectively, compared with 3.84 ± 0.21 in the sham-operated group, and the ANH-CH hematocrit 25% group had a significantly higher level than the other three groups (P<0.001; Figure 6).

Discussion

In our study, ANH-CH with low hematocrit (≤20%) caused cerebral hypoxic-ischemic injury in rats. It was also associated with increased NF κB activity and TNF-α level in the cerebral cortex. These data provide experimental evidence that ANH-CH with hematocrit ≤20% should be avoided.

ANH and CH are independently effective in decreasing operative blood loss and transfusion of allogeneic blood (2,3). Their combination may further reduce the need for allogeneic blood transfusion (17,18). However, a confirmed safe limit of ANH-CH has not been reported, and blood-sparing benefits of the combination must be weighed against the risk of inadequate tissue oxygenation (17).

In our study, ANH-CH did not cause serious hemodynamic instability. HR increased only moderately during CH, which might be a compensation mechanism for decreased blood pressure. In addition, at T4, the point of recovery after CH, MAP was lower than at baseline in all treatment groups, possibly because of hydroxyethyl starch 130/0.4 decreasing blood volume expansion, which occurred 2 hours after the infusion of hydroxyethyl starch. PaO2 did not differ from baseline levels during ANH-CH but showed a decreasing trend, especially in the ANH-CH hematocrit 15% group, which might result in the decreased pH. In addition, tissue hypoperfusion induced by CH might be another explanation for the decreased pH.

Hippocampal CA1 neurons are highly susceptible to short periods of transient global ischemia (7). We showed their significant alteration, verified with electron microscopy in rats hemodiluted to very low levels of hematocrit. Cerebral histological investigation of hippocampal CA1 neurons with electron microscopy revealed characteristic morphological damage, such as mitochondria denaturalization, nucleus distortion, and astroglial activation in severe ANH-CH (hematocrit ≤20%). It is clear that significant alterations in mitochondrial structure quickly occur in severely ischemic tissue (13). Also, several typical ultrastructural alterations in neurons, such as disruption of mitochondrial cristae, severe damage to mitochondrial membrane, and clumping and margination of chromatin in the
nuclei are related to irreversible neuronal injury (13). Similarly, we concluded that cerebral tissue was exposed to hypoxic-ischemic injury in ANH-CH with low hematocrit ($\leq 20\%$), which might have resulted from the combined reduction of oxygen-carrying capacity and perfusion pressure in the brain.

NF-$\kappa B$ in the brain is activated by various intercellular signals, including cytokines, neurotrophic factors, and neurotransmitters. In this study, NF-$\kappa B$ activities were increased in the cerebral cortex in rats treated with ANH-CH and reached the highest level in hematocrit 25% group, while the ultrastructural study of brain showed no obvious changes. For ANH with extremely low hematocrit groups, up-regulated activities of NF-$\kappa B$ were suppressed. Previous studies have shown that NF-$\kappa B$ is important for the induction of neuroprotective anti-apoptotic gene products such as MnSOD and Bcl-2, which are known to contribute to ischemic tolerance (6). In contrast to the anti-apoptosis action of NF-$\kappa B$, Clemens et al (7) have reported that transient forebrain ischemia resulted in a marked activation of NF-$\kappa B$ in the highly vulnerable CA1 sector, which may be associated with apoptotic or necrotic cell death. Xu et al (19) have indicated that acute inhibition of NF-$\kappa B$ activation reduced brain injury in a rat model of middle cerebral artery occlusion. Our results may suggest that hypoxia at doses close to but below the threshold of cell injury induce NF-$\kappa B$ activation as an adaptive response. While hypoxia reaches the threshold, NF-$\kappa B$ activities may be attenuated because of brain resident cells injury. We could not define whether NF-$\kappa B$ activation serves as a neuroprotective process or not. The exact role of NF-$\kappa B$ in brain ischemic injury remains to be explored.

Recent data suggest that several inflammatory cytokines may be synthesized and secreted by several central nervous system cell types including microglia, astrocytes, and neurons (20). These cytokines could influence both progression of injury and regulation of wound healing in the brain. In our study, TNF-$\alpha$ levels in the cerebral cortex were elevated in all ANH-CH groups as early as 3 hours after ANH-CH, peaking in the ANH-CH hematocrit 25% group. These results support the hypothesis that cytokines play a functional role in the acute post-ischemic period. The turning point of TNF-$\alpha$ may suggest that brain damage would happen if severe ANH-CH (hematocrit $\leq 20\%$) was performed. Some authors consider that TNF-$\alpha$ and other cytokines may be of extreme benefit and exert key roles in repair and regeneration of the injured brain tissue. TNF-$\alpha$ has been shown to act in a synergistic manner with interleukin (IL)-1$\beta$ to produce nerve growth factor (9) and prevent metabolic consequences of glucose deprivation in cultured cortical, septal, and hippocampal neurons (21). The vulnerability of cortical and striatal neurons to focal ischemic injury are increased in mice lacking TNF-$\alpha$ receptors (22,23). On the other hand, data also show that TNF-$\alpha$ may be an important mediator in neuronal apoptosis in vivo as well as in neuronal loss as a result of brain injury (10,24). Neutralizing TNF-$\alpha$ activity by administration of TNF-$\alpha$ binding protein or antagonist attenuated brain ischemic injury (25,26). The functional role of cytokines in the brain injury is still controversial. In our study, changes in cerebral activities of NF-$\kappa B$ were parallel with the production of TNF-$\alpha$, which may indicate that TNF-$\alpha$ and NF-$\kappa B$ are both involved in the brain “homeostatic” response to ischemic injury, and TNF-$\alpha$ is a potential candidate for the role of NF-$\kappa B$ activator.

In conclusion, our study indicated that sodium nitroprusside-induced CH did not result in cerebral injury with moderate ANH, but did so with severe hemodilution (hematocrit $\leq 20\%$) and that ANH-CH may result in cerebral hypoxic-ischemic injury, represent-
ed by characteristic morphological damages in the CA1 region of the hippocampus. The combination of severe ANH (hematocrit ≤20%) and CH should be avoided. Though the exact function of NF-κB and TNF-α in cerebral hypoxic-ischemic injury remains unclear, they may be important factors in brain tolerance in ischemic condition. Understanding how cytokines and transcription factors regulate the response to injury will facilitate the development of treatments for these injuries.

Acknowledgments
We thank Dr Genbao Feng for excellent technical assistance.

References
24. Gregersen R, Lambertz K, Finsen B. Microglia and...


Correction: Sefer et al: Effects of Renal Transplantation on Hearing and Ocular Changes in a Monozygotic Twin with Alport’s Syndrome: Comparison with Other Twin on Hemodialysis

To the Editor: In the article by Sefer et al “Effects of renal transplantation on hearing and ocular changes in a monozygotic twin with Alport’s syndrome: comparison with other twin on hemodialysis” published in the Croatian Medical Journal (1), my last name was misspelled. As this misspelling causes problems with the retrieval of the articles I co-authored from bibliographical databases, the correct information on the article should be: Sefer S, Trotic R, Lacmanovic V, Degoricija V, Ratkovic-Gusic I, Kes P. Effects of renal transplantation on hearing and ocular changes in a monozygotic twin with Alport’s syndrome: comparison with other twin on hemodialysis. Croat Med J. 2000;41:203-6.


Vesna Degoricija
vesna.degoricija@zg.htnet.hr